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Functional properties of the reconstituted phosphate carrier from bovine heart mitochondria: Evidence for asymmetric orientation and characterization of three different transport modes

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The phosphate carrier from bovine heart mitochondria was reconstituted into liposomes by the removal of detergent using hydrophobic ion-exchange columns. Reversible blocking of the carrier function during chromatographic steps was possible by the application of the inhibitor *p*-(chloromercuri)benzenesulfonate at low temperature. Thus, both forward and backward exchange experiments for kinetic characterization of P_i/P_i-antiport as well as the P_i/H^+-symport could be performed. The maximum rate of P_i/P_i-antiport was 90 μmol min^{-1} (mg protein)$^{-1}$. Only one single half-saturation constant (K_m) for phosphate was observed at each side of the membrane under antiport conditions, 1.8 mM at the external and 9.4 mM at the internal side. By comparing the K_m values at both sides of the membrane with the values found in intact mitochondria, a right-side-out orientation of the reconstituted phosphate carrier was concluded. Furthermore, the influence of various sulfhydryl reagents on the carrier was investigated. After modification with $HgCl_2$, the phosphate carrier reveals a third (nonphysiological) unidirectional transport mode. This was characterized by a significantly reduced substrate specificity. In view of similar observations with several other mitochondrial carriers, these results again indicate that the phosphate carrier is a member of the postulated functional family of mitochondrial carrier proteins.

Introduction

Uptake of inorganic phosphate into mitochondria is essential for energy metabolism. Two carrier systems are involved in phosphate flux across the inner mitochondrial membrane. The most important is the phosphate carrier (PIC), in addition the dicarboxylate carrier accepts phosphate as substrate (for review see Refs. 1–3). The PIC was purified and reconstituted in liposomes in a functional active state [4–13]. The amino acid sequence of the carrier was determined [14,15], together with the ADP/ATP-, the oxoglutarate carrier and the uncoupling protein; the PIC belongs to the structural family of mitochondrial carrier proteins [16].

The PIC catalyzes both P_i/P_i-antiport and P_i/H^+-symport, the physiologically important reaction being import of phosphate together with one proton [17]. Thus, phosphate import is electroneutral (H^+-compensated) and its activity is modulated by the pH-gradient across the inner mitochondrial membrane [1]. In contrast to detailed kinetic studies in the case of other mitochondrial (antiport) carriers, no conclusive analysis of the reaction mechanism of the PIC is available so far. Few attempts at a kinetic characterization of the PIC have been made in reconstituted systems [4–13]. Among other things, experimental problems caused by the use of freeze/thaw/sonication methods for functional reconstitution have hampered a conclusive kinetic evaluation.

In this paper we use the Amberlite method of reconstitution, which was originally established for the aspartate/glutamate carrier [18]. Since then most of the other mitochondrial carriers have been reconstituted by the use of this method [19,20]. Together with the development of an effective and reversible block of substrate efflux during chromatographic steps, we analyzed the basic kinetic properties of the PIC in the reconstituted system.

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, 1,4-dithioerythritol; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; NEM, *N*-ethylmaleimide; pCMB, *p*-(chloromercuri)benzoate; pCMBS, *p*-(chloromercuri)benzenesulfonate; PIC, phosphate carrier; PLP, pyridoxal 5′-phosphate.

It has been shown recently that at least three mitochondrial carrier proteins, i.e., the aspartate/glutamate carrier, the ADP/ATP carrier [21,22], and the carnitine carrier [23], can be reversibly converted from the physiological function of substrate antiport into uniport (efflux) systems by cysteine modification. The detailed analysis of this functional conversion induced by mercurials led to the interpretation of an intrinsic preformed channel structure as a common element in these carriers. It was thus interesting to investigate whether the PIC also belongs to this functional family.

Materials and Methods

Materials and their sources

[^{32}P]Phosphate, [^{35}S]sulfate, L-[U-^{14}C]aspartate, L-[U-^{14}C]glucose, [8-^{14}C]ATP, 2-oxo-[5-^{14}C]glutaratic acid, L-[U-^{14}C]lysine were obtained from Amersham-Buchler. Sigma supplied the following chemicals: cardiolipin, Triton X-114, mersalyl acid, pCMBS, pCMB, DTE, DTNB, Hepes, and turkey egg-yolk phospholipid. Amberlite XAD-2 and Dowex 1-X8 were purchased from Fluka, hydroxyapatite (Bio-Gel HTP) from Bio-Rad, Sephadex from Pharmacia, PLP, NEM and $HgCl_2$ from Merck. All further chemicals were of analytical grade.

All sulfhydryl reagents used ($HgCl_2$, mersalyl, 5,5′-dithiobis(2-nitrobenzoate), *p*-(chloromercuri)benzoate and *p*-(chloromercuri)benzenesulfonate), were prepared from frozen stock-solutions. The reagents were diluted with water or the respective gel filtration buffer. PLP in high concentrations was dissolved in 1 M imidiazole (pH 6.5), lower concentrations ($\leq$ 150 mM) were dissolved in the respective gel filtration buffer (20 mM Hepes, 30–100 KCl (pH 6.5)).

Preparation of proteoliposomes

The phosphate carrier was purified from frozen bovine heart mitochondria (prepared by the method of Smith [24]) as described by Bisaccia et al. [9,25]. In order to carry out experiments with low internal phosphate concentrations we had to reduce the phosphate in all solutions. On the other hand, optimum conditions for purification and reconstitution in general require the continuous presence of high substrate (phosphate) concentrations. We found that decreasing the phosphate concentration to 2 mM during the whole purification process had only a slight effect on the transport activity, if, simultaneously, 50 mM KCl was added to keep the ionic strength of the mediums at an appropriate level. Furthermore, we used 20 mM Hepes (pH 6.5) for a sufficient buffer capacity. In all cases the second protein fraction obtained after hydroxyapatite chromatography was used for reconstitution. The protein was incorporated into liposomes by hydrophobic chromatography of mixed micelles on Amberlite beads in a recycling procedure. The exact reconstitution conditions are described in the results section. In general the liposomes contain 20–30 mM internal phosphate, exceptions are given in figure legends.

Transport measurements

The reconstituted transport activities (antiport, symport or efflux) of the PIC were determined by measuring the flux of [^{32}P]phosphate. The applied methods resemble the techniques described previously for the kinetic analysis of the aspartate/glutamate carrier [21,26]. Antiport activity was determined by using two different modes based upon the inhibition-stop technique. In forward exchange experiments (uptake of label) the assay was started by the addition of labeled phosphate to proteoliposomes containing unlabeled countersubstrate inside. To measure the flux of phosphate in backward exchange mode (export of label), the internal pool was prelabeled by adding [^{32}P]phosphate of high specific radioactivity for 10 minutes. In experiments where the knowledge of the exact internal substrate concentration was required, [^{32}P]phosphate was added after reconstitution. In the other cases [^{32}P]phosphate was added to proteoliposomes previously separated from external phosphate by passage over an anion-exchange column (Dowex 1-X8, chloride form, 4°C).

Before the kinetic experiments, in general, the external substrate had to be separated by size exclusion chromatography on Sephadex G-75 columns. Since the PIC also catalyzes P_i/H^+-symport, the use of this method for separating external phosphate depends on the availability of an effective and reversible inhibitor. Experiments for the development of an appropriate procedure are described in the results section. Routinely, 5 μM pCMBS was used for the reversible inhibition of efflux during column chromatography which was performed at 4°C. When the nonphysiological efflux was investigated 1 mM NEM was applied for inhibition. After removal of external phosphate, transport was started by adding 5 μM DTE (P_i/H^+-symport) or DTE in combination with external phosphate (P_i/P_i-antiport). It has to be mentioned that under identical conditions the time-course of isotope equilibration measured in the backward transport mode was in perfect agreement with measurements in the forward transport mode, where no additional inhibitor was necessary. This demonstrates that the procedures needed for reactivation of the carrier had no influence on the time-course of the measured transport (results not shown). After the desired period of time the carrier-mediated transport was stopped by using high concentrations ($\geq$ 5 mM) of NEM, or, if DTE was present in the medium, with high concentrations ($\geq$ 25 mM) of PLP. The procedure for measuring unidirectional phosphate flux in the mercurial-induced efflux mode

resembled the backward exchange method. The export of label, however, was started by adding $\geqq 0.1$ mM $HgCl_2$. Transport was stopped by adding a mixture of DTE (5 mM) and PLP ($\geqq 25$ mM). Finally, after treatment with the stop inhibitor each sample was passed through an anion exchange column (Dowex 1-X8, chloride form) in order to remove the external radioactivity. All transport determinations were carried out at the same internal and external pH value of 6.5. Further experimental details concerning gel filtration chromatography, removal of external radioactivity on anion-exchange columns and evaluation of transport activities by computer fitting are given in Refs. 21 and 26.

Determination of protein concentration

The protein concentrations were determined according to the modified Lowry method after precipitation with deoxycholate and trichloroacetic acid and extracting detergent and lipid with organic solvents [27,28].

Results

Optimization of the reconstitution procedure and kinetic analysis

In order to improve the conditions for kinetic analysis of the reconstituted phosphate carrier, we did not use the freeze/thaw/sonication method applied in earlier studies [4–13]. Instead, we adapted and modified the Amberlite method for functional reconstitution [18] to the particular needs of the PIC. In experiments with other mitochondrial carriers, e.g., the aspartate/glutamate carrier [18], it has been observed that especially the phospholipid/protein ratio is a critical parameter during reconstitution in order to obtain maximum transport activity. Fig. 1 shows the influence of the amount of membrane-inserted protein on three basic parameters of carrier reconstitution. The specific exchange rate showed a sharp optimum between 0.9–1.5 μg protein/mg phospholipid. The addition of higher amounts of protein led to significantly lower specific transport rates. Whereas the total internal volume of the proteoliposomes sharply decreased with increasing amounts of added PIC, indicating reduction of the 'liposomal size', the volume of the functional active liposomes reached a maximum value. This maximum value of active internal volume could not be increased by increasing amounts of added PIC. The obtained protein/phospholipid ratio for optimal exchange activity equaled that observed for the carnitine carrier [29], whereas the optimum ratio measured for the ADP/ATP carrier or the aspartate/glutamate carrier [18] is about 10-fold and 100-fold higher, respectively.

Other parameters of the reconstitution procedure were also tested, e.g., the total amount of phospholipid, the detergent/phospholipid ratio, the detergent/Amberlite ratio or the number of column passages. Optimum values for these parameters were found to be in the same range as observed for the other mitochondrial carrier proteins. Maximum transport activity was obtained under the following conditions. We used 12 mg phospholipid/ml in combination with a detergent/phospholipid ratio of 1.6–1.8 mg/mg. The detergent/Amberlite ratio could be chosen between 15 mg/g and 30 mg/g. Removal of the detergent was achieved after about 15 column passages.

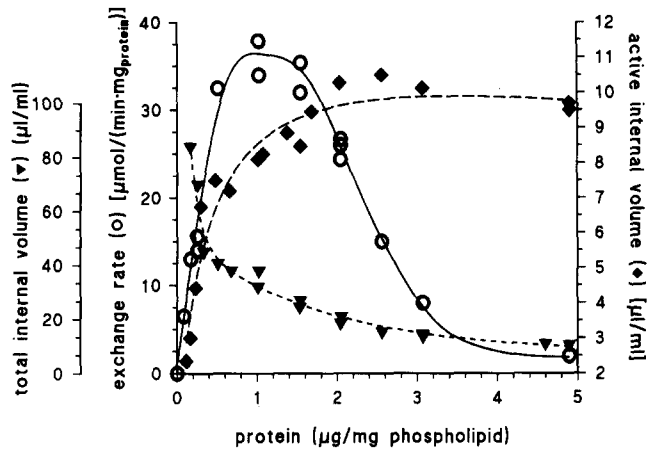

Fig. 1. Reconstitution of the phosphate carrier: dependence on the protein/phospholipid ratio. The constant reconstitution parameters were: phospholipid 12 mg/ml; Triton X-114/phospholipid 1.6 mg/mg; Triton X-114/Amberlite 20 mg/g; number of column passages: 15. The exchange was measured under the following conditions: external phosphate 1.5 mM, internal phosphate 20 mM (pH 6.5). The specific exchange activity (○) was derived from initial velocity measurements, whereas the active internal volume (◆) was calculated by the total amount of labeled phosphate taken up under equal phosphate concentrations at both sides of the membranes. The total internal volume (▼) was derived from the determination of phosphate and phospholipids [18].

The addition of cardiolipin during purification of the PIC is essential for optimum activity [9,30]. Thus, we tested whether addition of this phospholipid during the reconstitution procedure applied here enhances the PIC activity. No stimulating effect was observed at cardiolipin concentrations below 5% of total phospholipid, whereas higher concentrations decreased the activity significantly (results not shown).

The advantage of using the Amberlite method instead of the freeze/thaw/sonication method for reconstitution of the PIC is illustrated in Fig. 2. Under comparable (but suboptimal!) conditions, the Amberlite method led to the formation of proteoliposomes with at least two-fold higher active internal volume and higher specific exchange activity. More important for the following kinetic experiments was the fact that proteoliposomes reconstituted by the Amberlite method obviously showed a significantly lower value of nonspecific transport activity, indicated by the complete inhibition obtained after incubation with NEM.

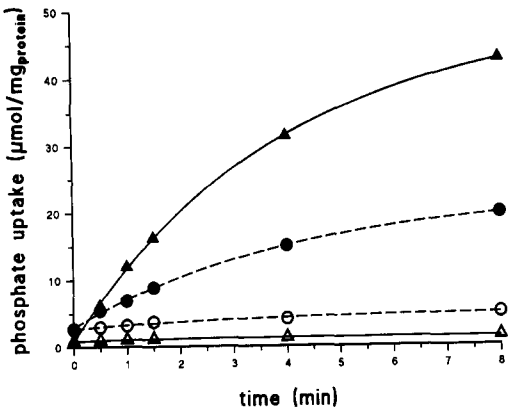

Fig. 2. Time-course of phosphate/phosphate exchange in liposomes formed by the Amberlite method (▲, △) or the freeze/thaw/sonication method (●, ○). 0.5 mM [^{32}P]phosphate was added to proteoliposomes containing 20 mM internal phosphate. The uptake catalyzed by the unmodified carrier (solid symbols) and after inhibition by preincubation with 2 mM NEM (open symbols) is shown. The following transport activities were determined: 12 μmol/min per mg (▲) or 0.21 μmol/min per mg (△) respectively; and 4.5 μmol/min per mg (●) or 0.75 μmol/min per mg (○), respectively.

The application of widely varying external (and internal) phosphate concentrations is essential for kinetic analysis. Thus the external phosphate has to be removed prior to the start of the kinetic experiment. Since the PIC catalyzes both P_i/P_i-antiport as well as P_i/H^+-symport the methods usually applied for the removal of external phosphate, i.e., size exclusion or ion exchange chromatography, cannot be used unless phosphate efflux is blocked during the chromatographic step. For this, a stop inhibitor is essential and must be both highly effective as well as reversible. Besides other inhibitors, which all proved not to be sufficiently reversible, various cysteine specific reagents were tested with respect to inhibition and to reversibility by DTE [31]. The results are shown in Table I. The data indicate that only pCMB or pCMBS in combination with low temperatures during chromatography are suitable for this purpose.

Basic kinetic data of the reconstituted phosphate carrier

By means of this method, we examined the efflux of [^{32}P]phosphate as P_i/H^+-symport (in the absence of external phosphate) or as P_i/P_i-antiport (in the presence of various external phosphate concentrations). In Table II the calculated first-order rate constants (k) of the recorded isotope equilibration are compared. The individual values are in the range between 0.55 (min^{-1}) (P_i/H^+-symport) and 1.61 (min^{-1}) (antiport in the presence of saturating external substrate concentrations). We proved that under conditions of 20 mM internal and 1.5 mM external phosphate unidirectional phosphate flux (P_i/H^+-symport) was very slow in comparison to the antiport reaction. Based on the fact that a maximum antiport rate of 90 μmol min^{-1} (mg protein)$^{-1}$ was measured in forward exchange experiments (see Fig. 3A), the rate for phosphate/H^+-symport activity (without external phosphate) can be calculated as 30 μmol min^{-1} (mg protein)$^{-1}$.

Exact determination of the substrate affinity of the PIC on both sides of the liposomal membrane is of importance in several respects. First the K_m value for internal phosphate in intact mitochondria is still unknown as is also true of the K_m value in the P_i/H^+-

TABLE I

Inhibition of P_i/H^+-symport by various sulfhydryl-reagents

The internal substrate pool was prelabeled by adding [^{32}P]phosphate at high specific radioactivity to proteoliposomes with 20 mM internal and external phosphate. After the addition of the inhibitor and passage over Sephadex columns (at 4°C), the remaining internal radioactivity was determined by liquid scintillation counting. In parallel, the dilution of liposomes during chromatography was measured. Both results together allow the efflux of internal substrate to be calculated during size exclusion chromatography. The reversibility of the reagent was determined by adding 5 mM DTE to the inhibited carrier and subsequent determination of the transport activity in forward exchange experiments.

Modification of the PIC with:	Efflux of internal substrate (%)	Reversibility of the reagent (%)
Buffer (H_2O)	≥ 60	–
5 μM pCMB	25	103
50 μM pCMB	2	112
1 μM pCMBS	30	104
5 μM pCMBS	≤ 3	98
50 μM pCMBS	1	101
50 μM DTNB	1	42
1 mM NEM	≤ 1	0
10 μM $HgCl_2$	8	98
100 μM $HgCl_2$	32	≈ 100 [a]
1 mM $HgCl_2$	≤ 78	≈ 92 [a]
100 μM mersalyl acid	9	105
1 mM mersalyl acid	14	95

[a] Exact determination of the reversibility was not possible because of efflux before measurement of antiport activity (see Results).

TABLE II

Discrimination between carrier-mediated P_i/P_i-antiport and P_i/H^+-symport

After prelabeling proteoliposomes with [^{32}P]phosphate and removal of external phosphate by size exclusion chromatography in the presence of 5 μM pCMBS, the activity of the two physiological activities of the phosphate carrier was determined by backward transport experiments. For better comparison of symport and antiport rates, apparent first-order rate constants k are given. In all cases, 5 mM DTE was added.

External phosphate (mM)	k (min^{-1})
none	0.55
1.5	0.62
5	0.89
30	1.61

symport mode. Furthermore, knowledge of these values can be used to determine the orientation of reconstituted carrier proteins embedded in the liposomal membrane [26,32]. In order to determine the K_m value for the P_i/P_i-antiport, both forward and backward exchange measurements had to be carried out. Fig. 3A shows the Eadie-Hofstee plot for exchange rates in the forward mode with variation of the external substrate between 0.1 and 37.5 mM at fixed internal phosphate (30 mM). The values in Fig. 3B were derived from backward exchange experiments with various internal phosphate concentrations (0.3–37.5 mM) and constant external phosphate (30 mM). The plots demonstrate the presence of only one single K_m value at each side. The external affinity for phosphate ($K_m = 1.8 \pm 0.1$ mM) was significantly lower than the internal affinity ($K_m = 9.4 \pm 0.5$ mM). Furthermore, we measured the internal K_m value in the P_i/H^+-symport mode using proteoliposomes with phosphate only in the internal space at concentrations between 0.3 and 37.5 mM (Fig. 3C). In these experiments transport was started by adding DTE, in order to remove the inhibitor pCMBS, in the absence of external phosphate. The calculated K_m value was 11.2 ± 0.9 mM, which is very close to the internal substrate affinity observed in the P_i/P_i-antiport mode.

TABLE III

Efflux induction by mercurial reagents

Different mercurials were added to prelabeled proteoliposomes with incorporated phosphate carrier, or to identical but carrier-free liposomes. In the latter case, [^{32}P]phosphate was already entrapped during reconstitution. Before removal of the external phosphate by size exclusion chromatography, the carrier had to be inactivated by pCMBS, DTNB or NEM (see Materials and Methods). Apparent first-order rate constants for the different experiments were determined. n.d., not determined.

First reagent	Second reagent	Efflux rate constant k (min^{-1})	
		proteoliposomes	pure liposomes
5 μM pCMBS	5 mM DTE	0.55	0.01
5 μM pCMBS	0.1 mM pCMBS	0.01	n.d.
5 μM pCMBS	1.0 mM pCMBS	0.01	0.01
5 μM pCMBS	2.5 mM pCMBS	0.01	0.01
5 μM pCMBS	0.1 mM mersalyl acid	0.01	n.d.
5 μM pCMBS	0.5 mM mersalyl acid	0.08	0.06
5 μM pCMBS	3 mM mersalyl acid	1.20	0.89
5 μM pCMBS	0.1 mM $HgCl_2$	0.28	0.01
5 μM pCMBS	0.25 mM $HgCl_2$	0.46	0.01
5 μM pCMBS	0.5 mM $HgCl_2$	0.65	0.02
5 μM pCMBS	1 mM $HgCl_2$	2.25	0.07
5 μM pCMBS	1.25 mM $HgCl_2$	2.42	0.06
5 μM pCMBS	4 mM $HgCl_2$	6.90	2.95
5 μM pCMBS	1 mM DTNB	0.01	n.d.
50 μM DTNB	1 mM $HgCl_2$	2.10	0.07
1 mM NEM	10 mM NEM	0.01	0.01
1 mM NEM	0.5 mM mersalyl acid	0.06	0.04
1 mM NEM	0.1 mM $HgCl_2$	0.45	0.02
1 mM NEM	1 mM $HgCl_2$	1.95	0.05

A third transport mode after modification by $HgCl_2$

It has been reported that modification of cysteine residues by mercurials like $HgCl_2$, mersalyl acid or pCMBS can convert the function of mitochondrial carrier proteins from counterexchange to unidirectional transport [21–23]. As already documented in Table I, preincubation with $\geqq 100$ μM $HgCl_2$ of proteoliposomes with reconstituted PIC leads to efflux of internal substrate. In Table III, the impact of various combinations of sulfhydryl reagents on these proteoliposomes and in parallel on protein-free liposomes was examined. In all experiments two different reagents had to be applied. The first reagent was necessary to inhibit depletion of the internal substrate pool during column chromatography. After addition of the second reagent, its potency with respect to efflux induction was tested. Only $HgCl_2$ was able to induce efflux of internal phosphate, other sulfhydryl reagents had no effect. The very fast efflux of internal phosphate after the addition of 3 mM mersalyl acid or 4 mM $HgCl_2$ was obviously caused by an increased permeability of the liposomal membrane. The resulting rate constant for the $HgCl_2$-induced efflux was more or less independent of the first reagent applied. This was thoroughly tested in the case experiments where 1 mM $HgCl_2$ was used as second reagent (in combination with pCMBS, NEM or DTNB). In this particular cases the rate constants in all experiments with different first reagents were determined with the same preparation of proteoliposomes; only under these conditions they can directly be compared [26].

Two facts support our interpretation that the efflux observed after incubation with $HgCl_2$ in concentrations $\leqq 1.5$ mM resembles the unidirectional transport mode already described for other mitochondrial carriers. First, efflux was strictly coupled to the inhibition of P_i/P_i-antiport and P_i/H^+-symport. This is demonstrated by the fact that efflux was also active in the presence of NEM, a well known inhibitor of the PIC [33–35]. Second, efflux induction caused by $HgCl_2$ in concentrations $\leqq 1.5$ mM was only observed in liposomes containing the reconstituted PIC.

Although the observed phosphate efflux was clearly protein-mediated, it was necessary to disprove the possibility of nonspecific fluxes through denatured carrier protein. Other inhibitors of the PIC, e.g., lysine or histidine specific reagents (e.g., phenylisothiocyanate [25], 4,4′-diisothiocyanostilbene-2,2′-disufonate (DIDS), PLP or diethylpyrocarbonate) could not be applied for this purpose. Although these reagents blocked both P_i/P_i-exchange and P_i/H^+-symport, they caused no reduction of $HgCl_2$-induced efflux (results not shown). On the other hand, the conversion to the

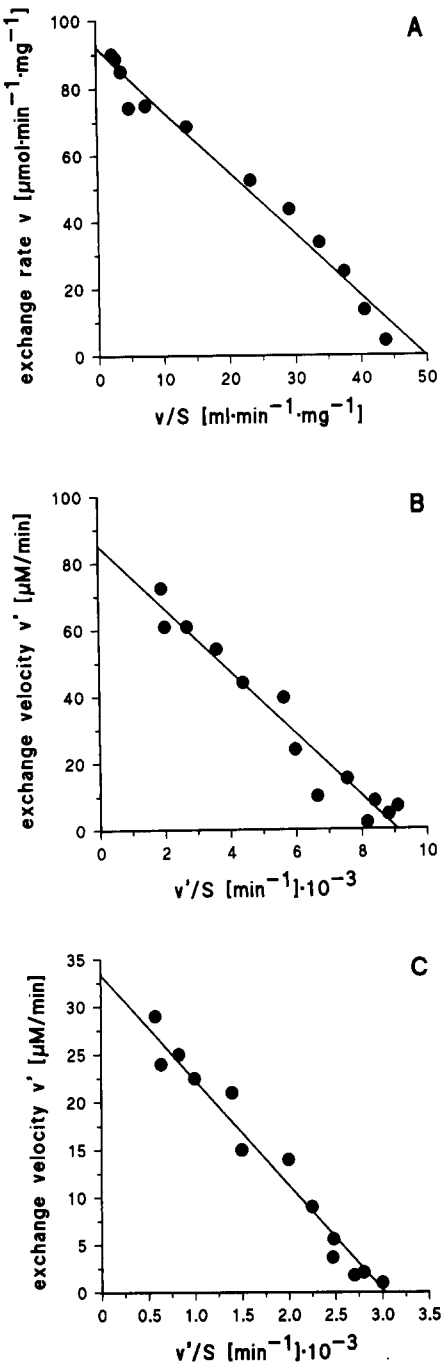

Fig. 3. Transport affinities of the reconstituted phosphate carrier. P_i/P_i-antiport measured at the external membrane side (A) and at the internal membrane side (B), as well as P_i/H^+-symport (K_m value of phosphate at the internal membrane side, (C)). Eadie-Hofstee plots were obtained from exchange experiments in the forward (A) and backward direction (B), as well as from efflux experiments (C). The substrate concentrations were as follows. 30 mM internal and 0.1–37.5 mM external (A), 30 mM external and 0.3–37.5 mM internal (B), and 0.3–37.5 mM, internal only (C), respectively. v' is defined as follows: $v' = k \cdot S_{in} \cdot V_{in}$; k is calculated by the equation: $\alpha = 100\,(1 - e^{-kt})$ [α: percentage of isotope equilibration, S_{in}: internal substrate concentration, V_{in}: internal volume of proteoliposomes catalyzing phosphate transport]. The K_m values derived are: external phosphate, 1.8 ± 0.1 mM; internal phosphate (antiport) 9.4 ± 0.5 mM; and internal phosphate (symport) 11.2 ± 0.9 mM.

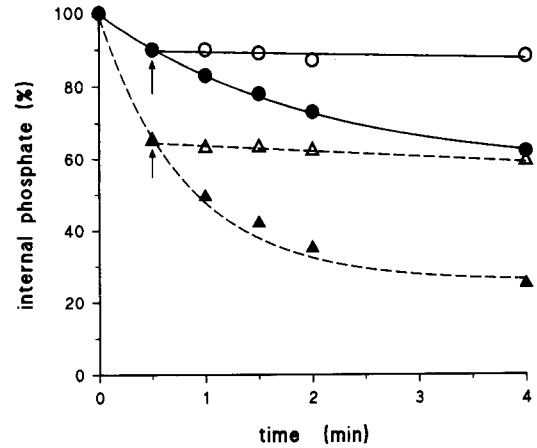

Fig. 4. Stop to $HgCl_2$-induced efflux by removing bound mercury with 5 mM DTE. Proteoliposomes, prelabeled with [^{32}P]phosphate, were incubated with 1 mM NEM (see Materials and Methods). After removal of external phosphate by size exclusion chromatography, efflux (closed symbols) of labeled phosphate was measured after adding 0.5 mM $HgCl_2$ (●) or 1.25 mM $HgCl_2$ (▲). In parallel, 0.5 min after induction of efflux 5 mM DTE was added (open symbols), as indicated by the arrows. The following first-order rate constants (k (min^{-1})) were calculated. 0.02 (○), 0.46, (●) 0.04 (△) and 1.3 (▲).

efflux mode was fully reversible as seen in Fig. 4. In this experiment, phosphate efflux was started by adding $HgCl_2$ in concentrations of 0.5 and 1.25 mM to proteoliposomes which had been previously inhibited with 1 mM NEM. In parallel, 0.5 min after the induction of efflux, 5 mM DTE was added, which led to an instantaneous cessation of phosphate efflux. Denaturation of the PIC at $HgCl_2$ concentrations $\leq$ 1.25 mM can thus be excluded. Another result which clearly argues against nonspecific leakage was obtained by measuring the influence of external phosphate on the rate of the $HgCl_2$-induced efflux, i.e., studying a kinetic trans effect of the substrate on efflux (Table IV). Addition of 1 mM external phosphate decreased the apparent rate

TABLE IV

Influence of external phosphate on efflux activity

Proteoliposomes were prelabeled with [^{32}P]phosphate. Before removal of the external phosphate by size exclusion chromatography, the carrier was inactivated by 1 mM NEM (see Materials and Methods). Efflux was induced by adding 0.25 mM or 2.5 mM $HgCl_2$ in the absence or presence of external phosphate. The apparent first-order rate constants k are given.

Induction efflux by	External phosphate concentration (mM)	k (min^{-1})
0.25 mM $HgCl_2$	–	0.61
	1	0.41
	25	0.33
2.5 mM $HgCl_2$	–	4.20
	1	4.05
	25	3.96

TABLE V

Efflux of various solutes catalyzed by the phosphate carrier after modification by $HgCl_2$

The labeled reagents were entrapped into proteoliposomes containing the purified phosphate carrier during reconstitution. Before removal of the external phosphate by size exclusion chromatography, the carrier was inactivated by 1 mM NEM (see Materials and Methods). After size exclusion chromatography we tested the passive membrane permeability for the solutes (addition of gel filtration buffer) or we induced the efflux mode of the phosphate carrier by adding 1 mM $HgCl_2$. Apparent first-order rate constants for all experiments were determined.

Entrapped solute	Apparent rate constant k (min^{-1}) after addition of	
	buffer	1 mM $HgCl_2$
[^{32}P]Phosphate	0.010	2.250
[^{35}S]Sulfate	0.015	0.720
[^{14}C]Oxoglutarate	0.020	0.250
[^{14}C]Glucose	0.160	0.490
[^{14}C]Aspartate	0.008	0.035
[^{14}C]ATP	0.018	0.023
[^{14}C]Lysine	0.013	0.030

constant by more than 30%, 25 mM external phosphate led to 50% reduction. This inhibition was hardly detectable at $HgCl_2$ concentrations $\geqq$ 2.5 mM, which is in agreement with nonspecific leakage of the membrane after incubation with high concentrations of $HgCl_2$.

As investigated in detail, especially in the case of the aspartate/glutamate carrier, mercurial-induced efflux catalyzed by reconstituted mitochondrial carriers combines both channel-like and carrier-like properties [20,22]. This pattern was also proven for the PIC. The results shown in Table V demonstrate that, in addition to the efflux of phosphate, anionic solutes like sulfate or oxoglutarate were translocated. The apparent first-order rate for the efflux of glucose was enhanced 3-fold by $HgCl_2$, as compared to the value based on diffusion alone (addition of buffer instead of $HgCl_2$). In contrast, no significant efflux of amino acids (aspartate or lysine) or of ATP was observed.

Discussion

It was necessary for this and the following kinetic analyses of the phosphate carrier to establish a reconstituted system with high activity and high liposomal (internal) volume. Since the freeze/thaw/sonication procedure so far used [4–13] did not meet these demands, we developed an optimized Amberlite method for reconstitution of the PIC, a procedure using detergent removal on hydrophobic ion-exchange columns [18]. The application of this procedure resulted in high transport rates of about 90 μmol min^{-1} (mg protein)$^{-1}$. This value is about 3-fold higher than the transport rates determined by Kaplan et al. [13] or Mende et al. [12]. A similar value of 71 μmol min^{-1} (mg protein)$^{-1}$ was determined by Palmieri et al. [36,37] using the freeze/thaw/sonication method. It has to be mentioned that Müller et al. [38] observed that the PIC purified from fresh bovine heart mitochondria (used in Refs. 36,37) in general gives higher net transport rates than the carrier purified from frozen mitochondria (used in the present study). As shown in Fig. 1, the main advantage of the Amberlite preparation was a considerable reduction of nonspecific transport phenomena.

Furthermore a system had to be developed permitting phosphate flux to be measured in backward transport experiments under controlled internal phosphate concentration. This was possible by the use of the inhibitors pCMB or pCMBS, to avoid the efflux of internal phosphate during size exclusion chromatography on Sephadex G-75. Both reagents block antiport and uniport activity of the PIC totally at micromolar concentrations, they are completely reversible by DTE, and they do not induce the nonphysiological efflux activity.

By measuring the efflux of [^{32}P]phosphate from prelabeled proteoliposomes under different conditions, we determined a maximum transport rate of 30 μmol min^{-1} (mg protein)$^{-1}$ for the P_i/H^+-symport mode. This rate is 3-fold higher than that determined by Wohlrab and Flowers [7], whereas in the paper by Guérin et al. [39] a transport rate of 70 μmol min^{-1} (mg protein)$^{-1}$ for P_i/H^+-symport catalyzed by the reconstituted PIC from *Saccharomyces cerevisiae* was derived.

In this optimized reconstituted system it was possible to determine the substrate affinity at both sides of the membrane for P_i/P_i-antiport. We observed only one single substrate affinity at each side, the external substrate affinity (1.8 mM) being significantly lower than the internal K_m value (9.4 mM). The substrate affinity of the PIC in mitochondria towards externally added phosphate is 1.6 mM [17,40]. Since a partial denaturation of the reconstituted protein would not lead to a decrease of substrate affinity solely on one side, the observation of two clearly distinct K_m values at both sides of the membrane can only be explained by assuming an asymmetric orientation of the reconstituted PIC. These findings lead to two conclusions. When comparing the determined K_m values with the published data obtained in mitochondria, a right-side-out orientation can be concluded, at least for that part of inserted phosphate carrier proteins which are functionally active and thus monitored by the kinetic experiments. Unfortunately, side-specific inhibitors of the PIC which would help to prove the predicted unidirectional orientation are not available. Furthermore, based on these measurements (see above) we conclude that

the internal K_m value for phosphate is about 10 mM. This value correlates well with estimations of free intramitochondrial phosphate, which are in the range of at least 6 mM [41]. An asymmetric orientation after reconstitution has also been reported for other mitochondrial carriers, e.g., for the aspartate/glutamate carrier [26,42], the oxoglutarate carrier [32] and the uncoupling protein [43]. All these mitochondrial carriers are inserted in right-side-out direction, when using a reconstitution method depending on the removal of detergent on Amberlite XAD-2 beads or similar materials. Therefore, the slow removal of the detergent obviously leads to a defined protein orientation.

Besides analyzing the substrate affinity for P_i/P_i-antiport, the reconstituted system also allowed us to determine the internal K_m value for the P_i/H^+-symport mode of the carrier as 11.2 ± 0.9 mM. It turned out that the internal substrate affinity for both physiological transport modes is more or less identical. The same observation was made in the case of the internal K_m values of the reconstituted carnitine carrier [29,44].

In the course of the experiments to establish the backward transport mode, we first obtained evidence of a possible third transport function of the PIC when incubating the proteoliposomes with $HgCl_2$ (Table I). This (nonphysiological) uniport mode caused by the modification of particular cysteine residues was first described for the aspartate/glutamate carrier and the ADP/ATP carrier [21,22] and later also for the carnitine carrier [23]. These observations were interpreted as indicating a preformed intrinsic channel within the carrier protein, which may possibly be a common motif for all secondary carrier proteins [20]. Interestingly, in the case of the reconstituted PIC, only $HgCl_2$ was able to induce efflux, whereas mersalyl acid or pCMBS, in contrast to the situation with other carrier proteins [21–23], were not effective. This finding may be rationalized assuming that in this case only the very small and hydrophilic $HgCl_2$ molecule can reach the reactive cysteine. Several arguments prove that the $HgCl_2$-induced phosphate efflux mode cannot be explained by nonspecific leakage of the proteoliposomes. Besides the fact that concentrations below 1.25 mM did not cause efflux of [^{32}P]phosphate in protein-free liposomes and partial denaturation could be ruled out (Fig. 4), further results strongly argue for the PIC as the mediator of the $HgCl_2$-induced uniport activity. First, efflux of internal phosphate was significantly reduced in the presence of external phosphate. The same effect was previously described for the aspartate/glutamate carrier [22]. Second, efflux induction was strictly coupled to inhibition of the P_i/P_i-antiport and the P_i/H^+-symport activity. Third, efflux could only be induced by $HgCl_2$ and not by other mercurials reacting at identical sites. It should be emphasized that, even in intact mitochondria, phosphate efflux not inhibitable by thiol reagents has been observed [45]. These findings were interpreted in stating that 'the inhibitor-insensitive phosphate efflux may occur in a portion of the PIC molecules that exist in a state different from that normally catalyzing phosphate influx' [45]. This interpretation is obviously correct and is fully explained by the results presented here.

The mechanism of efflux induction in the case of the PIC resembles that observed for the aspartate/glutamate carrier [22] and the carnitine carrier [23]. In these two papers, induction of the efflux mode was described as a result of the modification of two distinct sulfhydryl groups. Also in the present case, we have to assume the involvement of at least two different cysteine residues. All applied sulfhydryl reagents react with the cysteine residue at position 42, as was shown previously [46,47]. This modification causes inhibition of P_i/P_i-exchange and P_i/H^+-symport. In addition, $HgCl_2$ obviously reacts with further cysteine(s) (possibly cysteine 27, see below), leading to the induction of the efflux mode. As pointed out before, the apparent first-order rate constant for the induced efflux mode was independent of the first sulfhydryl-reagent applied to inhibit the symport reaction. This finding in particular resembles the situation observed for the antiport-efflux conversion of the reconstituted aspartate/glutamate carrier [22,48]. In that case, one particular cysteine could be made responsible for inhibition of the physiological carrier function, whereas additional modification of another cysteine was found to cause the uniport (efflux) mode. One can speculate that this additional cysteine may be the residue at position 27 of the beef heart phosphate carrier. This cysteine residue, located in the first hydrophobic segment, is the only conserved cysteine when yeast and bovine heart phosphate carrier are compared [15,49]. The yeast phosphate carrier which contains only three cysteine residues, is insensitive against inhibition by NEM since it lacks Cys-42; however, it still shows high sensitivity against mercurial compounds [39].

Unfortunately, no inhibitor for the phosphate efflux was found in the case of the PIC. Lysine and histidine specific reagents, like PLP or diethyl pyrocarbonate, inhibited the two physiological transport modes, whereas the $HgCl_2$-induced efflux mode was not affected. We tentatively explain this result, by analogy to the detailed investigations on the aspartate/glutamate carrier [21,22], by assuming that these positively charged amino acid residues are necessary for the gating process within the catalytic cycle of carrier function. After induction of the efflux activity, the carrier function is changed to a simpler transport mode, which becomes obvious by a significantly reduced substrate specificity. This nonphysiological transport mode does not depend on the functional presence of certain charged amino acid residues for the gating mechanism.

These results concerning the induction of a third transport activity by modification of the carrier with $HgCl_2$ support the hypothesis that the induction of this kind of uniport is a common phenomenon of mitochondrial carrier proteins [22,23]. This finding again argues for the fact that the functional family of mitochondrial carriers, besides showing sequential mechanism in transport catalysis [20,37,42], is also characterized by the common principle of an nonphysiological uniport activity inducible by modification of certain cysteine residues.

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